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DESIGNATED / ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371**

ATTORNEY'S DOCKET NUMBER

P66378US0

US APPLICATION NO. (known) 371 (2FF) (15)

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INTERNATIONAL APPLICATION NO.

PCT/EP00/02174

INTERNATIONAL FILING DATE

13 March 2000

PRIORITY DATE CLAIMED

12 March 1999

TITLE OF INVENTION

CHEMOSENSITIVITY MEASUREMENT USING CASPASE ACTIVITY

APPLICANT(S) FOR DO/EO/US

Franz Josef MEYER-ALMES

**Applicant herein submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.**

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for Internatl. Preliminary Examination was made by the 19th month from earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☒ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the Internatl. Preliminary Examination report under PCT Article 36 (35 U.S.C. 371(c)(5)).

**Items 11. to 16. below concern other document(s) or information included:**

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.  
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:

International Search Report — EPO

PCT/IB/301 Form

PCT/IB/304 Form

PCT/IB/308 Form

First Page of Publication

International Preliminary Examination Report — No Annexes

US APPLICATION NO. (if known, see 37 CFR 1.5)

09/762304

INTERNATIONAL APPLICATION NO

PCT/EP00/02174

ATTORNEY'S DOCKET NUMBER

P66378US0

17. ☒ The following fees are submitted:**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Internatl. prelim. examination fee paid to USPTO (37 CFR 1.492 (a) (1)) .. \$690.00

No international preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (2)) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .. \$710.00

Neither international preliminary examination fee (37 CFR 1.492 (a) (3)) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO) ..... \$1000.00

International preliminary examination fee paid to USPTO (37 CFR 1.492 (a) (4)) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... \$100.00

Search Report prepared by the EPO or JPO (37 CFR 1.492 (a) (5)) ..... \$860.00

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$ 860.00

Surcharge of \$130.00 for furnishing the **oath or declaration** later than☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

**Claims****Number Filed****Number Extra****Rate**

Total Claims

11 - 20 =

-0-

x \$18.00

\$

Independent Claims

2 - 3 =

-0-

x \$80.00

\$

Multiple Dependent Claim(s) (if applicable)

+ \$270.00

\$

**TOTAL OF ABOVE CALCULATIONS =**

\$ 990.00

Reduction by 1/2 for filing by **small entity**, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).

\$

**SUBTOTAL =**

\$ 990.00

Processing fee of \$130 for furnishing the **English translation** later than☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))

\$

**TOTAL NATIONAL FEE =**

\$ 990.00

Fee of \$40.00 for recording the enclosed **assignment** (37 CFR 1.21(h)).

Assignment must be accompanied by appropriate cover sheet (37 CFR 3.28, 3.31).

\$

**TOTAL FEES ENCLOSED =**

\$ 990.00

Amt. to be refunded:

\$

Amt. charged:

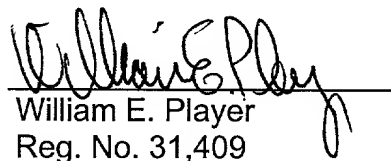
\$

- a. ☒ A check in the amount of \$ 990.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. 06-1358 in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge my account any additional fees set forth in §1.492 during the pendency of this application, or credit any overpayment to Deposit Account No. 06-1358. A duplicate copy of this sheet is enclosed.

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By

  
 William E. Player  
 Reg. No. 31,409

09/762304

JC05 Rec'd PCT/PTO 0 8 FEB 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Franz MEYER-ALMES  
Serial No.: New  
Filing Date: February 8, 2001  
For: CHEMOSENSITIVITY MEASUREMENT USING CASPASE ACTIVITY

PRELIMINARY AMENDMENT TO LESSEN FEES

Assistant Commissioner of Patents  
Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-identified application as follows:

IN THE CLAIMS

Claim 3, line 1, delete "any of claims 1 to 2",  
insert --claim 1--;  
Claim 4, line 1, delete "any of claims 1 to 3",  
insert --claim 1--;  
Claim 5, line 1, delete "any of claims 1 to 4",  
insert --claim 1--;  
Claim 6, line 1, delete "any of claims 1 to 5",  
insert --claim 1--;  
Claim 7, line 1, delete "any of claims 1 to 6",  
insert --claim 1--;  
Claim 8, line 1, delete "any of claims 1 to 7",  
insert --claim 1--;  
Claim 11, line 1, delete "any of claims 9 and/or 10",  
insert --claim 9--.

REMARKS

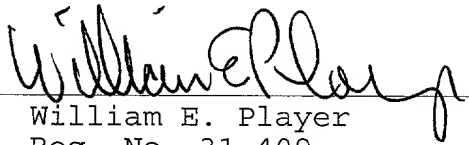
The foregoing Preliminary Amendment is requested in order to delete the multiple dependent claims and avoid paying the multiple dependent claims fee.

Early action on the merits is respectfully requested.

Applicants: Franz MEYER-ALMES

Respectfully submitted,

JACOBSON, PRICE, HOLMAN & STERN, PLLC

By   
William E. Player  
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Atty. Docket: P66378US0  
Date: February 8, 2001  
WEP:jrc

09/762304

JC05 Rec'd PCT/PTO 0 8 FEB 2001

SMB

### Chemosensitivity Measurement Using Caspase Activity

The present invention relates to a method for determining the chemosensitivity of cells towards at least one substance by measuring the apoptosis induced by said at least one substance.

## Chemosensitivity tests

Successful treatments and healings of tumor diseases have greatly increased in number since the introduction of chemotherapy. For example, the survival rate of infantile acute lymphatic leukemia (ALL) was less than 10% in the middle of the 1960s. Today, the chance of healing is above 70%. The medicaments, so-called cytostatic agents, are administered according to particular therapeutic plans alone or in combination with other active substances. By now, there are an immeasurable number of different therapeutic plans which have been established empirically and are constantly being developed further. The main criterion for an improved therapeutic plan is an improved survival rate (clinical outcome). This criterion is met by the majority of the patients. However, each individual has a different furnishing of cells with different properties. This is true, in particular, of the properties of tumor cells. In some studies, it could be shown that a therapy has an extremely good effect for one subpopulation while it is little effective or not effective at all for other patients because of drug-resistant tumors (Lacombe et al., Blood, 84, 716-723 (1994), Smit et al., International Journal of Cancer 51, 72-78 (1992)). It could be shown that not only the effectiveness of different medicaments but also the effective dose of one medicament can be different individually. Establishing the individual dosage of a medicament is important to patients in order to receive, on the one hand, as much medicaments as necessary for being healed and to keep, on the other hand, the toxic effects of the therapy and the

probability of a secondary cancer disease induced by the chemotherapy as low as possible. Good as the currently usual therapeutic plans may be, progress in terms of average survival rate has been stagnating in recent years. A further significant improvement of chemotherapy should be possible by establishing individually matched therapeutic plans.

To approach these problems, some researchers have attempted to measure the individual sensitivity of patients' tumors towards cytostatic agents in vitro. Most chemosensitivity tests currently used are at least partially based on the agar tumor culture test developed by Salmon (Salmon et al., Science, 197, 461-463 (1977)). Such tests measure the proliferation of the cells.

A second type of chemosensitivity test comprises the exclusion of (fluorescent) dyes or the release of the radioactive chromium isotope  $^{51}\text{Cr}$  which measures the disruption of the cell membrane by the direct or indirect action of cytostatic agents. A modification of the earlier dye exclusion test is the so-called DiSC assay (Weisenthal, Kern, Oncology 5: 92-103 (1991)) which additionally differentiates between normal and tumor cells by a second dyeing process.

The third type of chemosensitivity tests determines parameters of cellular metabolism as a measure of damage done by cytostatic agents. This test type comprises the radiometric BACTEC test (von Hoff D., Forseth B., Warfel L., in Salmon Trent (Ed.), Human tumor cloning, pp. 656-657, Grune & Stratton, Orlando (1984)), the MTT test (Freund A. et al., European Journal of Cancer 34: 895-901 (1998), Kaspers G.J.L., Blood 92: 259-266 (1998), Pieters R. et al., Leukemia 12, 1344-1348 (1998), Klumper E. et al., British Journal of Haematology 93: 903-910 (1996), Hwang W.S. et al., Leukemia Research 17: 685-688 (1993)) and its variations, the ATP test (Kangas L., Gronroos M., Nieminen A., Medical Biology 62: 338-343 (1984)) and the so-called FCA test (Meitner P., Oncology 5: 75-81 (1991), Rotman B., Teplitz C., Dickinson K., Cozzolino J., Cellular and Developmental Biology 24: f1137-1146 (1988)).

Agar culture assays have a great disadvantage in that not all tumor cells by far will grow in the agar cultures. This is true, in particular, of lymphatic leukemias

and lymphomas (Veerman A.J.P., Pieters R., British Journal of Haematology 74: 381-384 (1990)). In the best known representative of this assay type, the clonogenic assay, the percentage of cell populations which can be evaluated is as low as 30-40%. The cells have to grow for a very long time (10-20 days) before the evaluation is done. In addition, the expenditure of work is immense.

The dye exclusion tests and the  $^{51}\text{Cr}$  release test determine the proportion of cells having a defect cell membrane. These tests often include the fixation and subsequent microscopical evaluation of the cells or the measurement of the radioactivity in the supernatant. Since a rather rough parameter, the integrity of the cell membrane, is measured, such tests are non-specific and cannot distinguish between a specific anti-tumor activity of a substance and physical cell damage or cell damage caused by a substance, for example, by heavy erosion or oxidation. Consequently, test of this type give a positive result also with substances which generally cause damage to all cells and thus are not suitable as an anti-tumor medicament. The probably most telling test of the dye exclusion type is the DiSC test. In contrast to other dye exclusion tests, it is capable of differentiating between cell damage to tumor cells and to normal cells due to double staining. However, the double staining and subsequent microscopical evaluation are extremely time-consuming. In addition, the results vary as a function of the person performing the evaluation and the detail image observed with the microscope.

To radiometric tests, such as the  $^{51}\text{Cr}$  release test, the following applies quite generally: The use of radioactive isotopes in diagnostic tests is increasingly being avoided today because of the potential danger and the problems of disposal. In addition, by now, fluorescence and luminescence techniques can achieve the same sensitivities for a total duration of the test which is often even shorter.

Of the chemosensitivity tests making use of the metabolism of a cell as a measure of proliferation, the MTT test and its variations are used the most often. After 4 days of incubation of the cells with various cytostatic agents, the MTT test has a relatively short test duration of about 4 hours. In addition, 96 samples

can be evaluated in parallel in a microtitration plate using an ELISA reading device. This ensures an acceptable throughput with a relatively low personnel expenditure. The MTT test is based on the conversion of 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide into a blue formazan product. The conversion is catalyzed by dehydrogenases which are active only in living cells. The MTT test is dependent on a constitutive basic activity of the cells examined. However, it has been found that some cell types, e.g., particularly often some FAB subtypes of acute myeloid leukemia, have a highly reduced dehydrogenase activity (Santini V. et al., Hematological Oncology 7: 287-293 (1989)). Therefore, the chemosensitivity of these tumor cells cannot be evaluated with an MTT test. Another problem is the water-insoluble formazan crystals which form. They must be solubilized by the addition of organic solvents, such as dimethyl sulfoxide or isopropanol. Former experience has shown that this causes problems with the reproducibility of the tests.

## **Apoptosis**

From the works of various research groups (e.g., Sen S. et al., FEBS Lett. 307: 122-127, Darzynkiewicz et al., Cytometry 13: 795-808 (1992), Fulda S., Los M., Friesen C., Debatin K.M., Int. J. Cancer 76: 105-114 (1998)), it appears likely that cell death caused by cytostatic agents generally involves the mechanism of apoptosis. Apoptosis is a cell death programmed by the cell itself which can be induced by physiological factors in the organism or by chemicals, such as cytostatic agents. The programmed cell death plays an extraordinarily important role in the cytostasis, e.g., of the immune system. Thus, for example, T cells directed against endogenous structures are removed from the organism by apoptosis. Otherwise, symptoms of an autoimmune disease may appear, e.g., lupus erythematosus, arthritic diseases, or disease from a T-cell tumor. Substances like the tumor necrosis factor alpha, which is synthesized by macrophages, are natural indicators of apoptosis.

Apoptosis proceeds according to a programmed uniform scheme in which particular cysteine-containing proteases (caspases) are activated (Cohen G.M., Biochem. J. 326: 1-16 (1997)). Caspases are activated exclusively by apoptosis and therefore



are a specific parameter for the quantification of apoptosis. Up to the time of apoptosis, the cell membranes are still intact and impermeable to DNA-binding fluorescent dyes, such as propidium iodide. Therefore, caspase activity is a parameter for the activity of cytostatic agents which appears substantially earlier than the disruption of the cell membrane, which occurs hours later.

In addition to cell death by apoptosis, there is another form of cell death, that is necrosis, which is induced, e.g., physically, by osmotic shock, erosion or oxidation. This is manifested by a heavy degenerative damage to the cell.

Medenica (US 5,736,129) determines the extent of cellular damage from cytostatic agents by staining the cells with propidium iodide, followed by counting the stained cells in a FACS (fluorescence-activated cell sorter). However, this approach has the great disadvantage of not precisely differentiating between apoptosis and necrosis; consequently, it cannot make use of the specific mechanism of action of cytostatic agents. In addition, the number of cells required for a telling analysis is very high.

Caspase activity tests are most suitable for the specific detection of apoptosis since necrotic damage, which is not relevant to cytostatic effects, is not covered (see Table 1). The methods used in recent years for measuring caspase activity comprise the gel-electrophoretic separation of specific protein substrates as well as chromogenic and fluorogenic tests in which colored products are formed by the caspases (see, e.g., Cohen G.M., *Biochem. J.* 326: 1-16 (1997), Stennicke H.R., Salvesen G.S., *J. Biol. Chem.* 41: 25719-25723 (1997)). All these tests are characterized in that the cells are first sedimented at a particular time after the induction and washed with a buffer, followed by disruption with a lysis buffer for the caspases, which had previously been present in the interior of the cell, to be able to convert the added substrate.

Thus, a catalogue of Clontech, Palo Alto, California, Catalogue 98/99, pages 71-72 (1998), describes a method for the determination of the apoptosis of cells in response to the action of substances. In this experimental design too, washing steps are performed according to the relevant protocols.

WO-A-99/18856 relates to a method for measuring the chemosensitivity of cells towards substances using cell-permeating fluorogenic substrates. There is a drawback in that only special substrates can be employed.

Fulda S. et al. disclose a method for measuring doxorubicin, cisplatin and VP-16, which induce apoptosis, using flow cytometry. Thus, cell lysates cannot be measured. In addition, flow cytometry is a comparably tedious method.

WO-A-98/13517 relates to a method for the estimation of the extent of apoptosis in cells. Thus, the cells are loaded with a substrate for proteases of the ICE/Ced-3 family, and the conversion of the substrate by the protease is followed by flow cytometry. Thus, however, cell lysates cannot be measured. As stated above, flow cytometry is relatively tedious. In addition, the loading of the cells requires that the cells be permeabilized. This may result in complications. Often, from the permeabilization treatment, the cells are no longer sufficiently authentic, but rather correspond to an artificial system.

However, the following problem arises from the washing procedure in view of a simple and quick performance of the test requiring little cellular material:

Apoptosis and thus the development of caspase activity is a dynamic process in which the cell proceeds to the so-called secondary necrosis after some time. Therefore, a washing procedure removes the caspases of these destroyed cells.

Thus, a signal for cells currently undergoing apoptosis is obtained, whose number will decrease sometime by secondary necrosis. It is known that the maximum of the number of apoptotic cells depends on both the cytostatic agent examined and the cell type. It is not known a priori at which time the caspase activity and thus the extent of apoptosis must be measured. It follows that, in principle, several samples with, e.g., tumor cells subjected to different incubation times must be assayed for chemosensitivity for every patient. The need of primary cells of patients would then be so high that the chemosensitivity towards only one or a few cytostatic agents could be measured. In addition, the time consumption and

personnel expenditure for the chemosensitivity testing of a single patient would be extremely high.

It has been the object of the present invention to overcome the mentioned drawbacks of the prior art.

This object is achieved by a method for determining the chemosensitivity of cells towards at least one substance by measuring the apoptosis induced by said at least one substance, wherein the apoptosis is determined from the accumulated caspase activity of a sample comprising cells and a medium by adding said at least one substance to the sample followed by incubation, and measuring the accumulated caspase activity in the sample upon disruption of the cells without previously separating off the cells. In particular, the method according to the invention permits the use of substrates which are hardly cell-permeating or not cell-permeating at all.

It is essential that the accumulated caspase activity is measured. In this approach, cells, e.g., tumor cells of a patient, are incubated with at least one substance (e.g., cytostatic agent) for an extended period of time so that all effective pharmaceuticals, even the slowly acting, have had enough time to induce apoptosis. Subsequently, for example, a substrate present in a lysis buffer is added, and after the cell lysis has been effected, the total caspase activity formed, i.e., the accumulated activity, of the cellular suspension is measured. It has been found that caspases activated by cytostatic agents remain active even after the secondary necrosis of apoptotic cells, and that the accumulated caspase activity increases with incubation time and remains on a high level from the time when secondary necrosis increasingly occurs. The conventionally determined (with a washing step) caspase activity increases at the same rate as the accumulated caspase activity, but decreases when the secondary necrosis begins (see Figure 1). Thus, the principle of measuring the accumulated caspase activity permits to detect the effect of substances on the cells by a single caspase activity measurement. This test has a low need of cells and can be performed as a routine test by laboratory or technical assistants within a short time. Due to the easy automation, the personnel expenditure for performing the test is low. Since the test can be performed on ELISA

reading devices, which are available everywhere, no investment in equipment is necessary. Miniaturization is possible to further reduce the consumption of materials. It could be shown that cells can be kept in culture for some days in less than 10 µl of medium. The reduction of the number of cells per sample compartment from 100 to 1000 per test enables highly parallel analyses of samples with extremely few cells from patients, e.g., from fine needle biopsies. The low need of cells also permits the high throughput screening (HTS) of unknown substances for anti-tumor activity.

When the chemosensitivity of cells from pathological tissues is tested using caspase activity, it is advantageous to include in the test healthy cells from the tissue as well as the same cells with no added substance as references. In particular, it is appropriate to include in the test as a control a permanent cell line whose chemosensitivity is known, in order to document the functionality of the test and realize mistakes in the test performance.

The following substances, for example, may be used as said substances:

#### A) Cytostatic agents

Abrin, amethopterin, acivin, aclacinomycin A, alanine mustard, altretamine, aminogluthethimide, aminopterin, amsacrin (mAMSA), various anabolic steroids, anthrapyrazole, L-asparaginase, 5-axacytidine, Bacillus-calmette-guerin, bisantrene, busereline, busulfane, butyryloxyethylglyoxaldithiosemicarbazone, camptothecine, carbamate ester, carzinophyllin, CCNU, chlorambucil, chloroethylmethylcyclohexylnitrosourea, chloroethylcyclohexylnitrosourea, chlorodeoxyadenosine, corticotropin, cyproterone acetate, chlorotrianisene, chlorozotозine, chromomycin A, cytosine arabinoside (Ara-C), BCNU, bleomycin, cisplatin, carboplatin, cladribine, cyclophosphamide, dactinomycin, daunomycin, daunorubicin, decarbacin, doxorubicin, DTIC, dehydroemitine, 4-demethoxydaunorubicin, demothodoxorubicin, deoxydoxorubicin, dexamethasone, dibromodulcitol, dichloromethotrexate, diethylstilbestrol, bis(2-chloropropyl)-DL-serine, doxifluridine, elliptinium acetate, 4'-epidoxorubicin, epirubicin, epoietin-α, erythropoietin, esorubicin, estradiol, etoposide, fluoxymesterone,

flutamide, folic acid, fotemustine, ftorafur, 4-FU, fludarabine phosphate, 5-FU, floxuridine, galactitole, gallium nitrate, gosereline, G-CSF, GM-CSF, hydra, hexamethylmelamine (HMM), hydrocortisone, hydroxyprogesterone, 4-hydroperoxycyclophosphamide, ICRF 159, idamycin, ifosfamide, immunoglobulin IGIV, interferon, cobalt-protoporphyrin complex, leucovorin calcium, leuprolide, levodopa, levothyroxine, lindane, liothyronine, liotrix, lomustine, levamisole, masoprocol, maytansine, menogaril, 6-mercaptopurine, methosalene, methyl-esterone, methyllomustine, mithracin, mithramycin, mitotane, mitoxanthrone, methotrexate (MTX), 6-MP, mechlorethamine hydrochloride, medroxyprogesterone, megestrol acetate, melphalan, mesna, mitomycin C, nandrolone, sodium phosphate P32, navelbine, neocarzinostatin, nitrofurazone, nHuIFN $\alpha$ , nHuIFN $\beta$ , nHuIFN $\gamma$ , octreotide acetate, ondansetron hydrochloride, disodium pamidronate, pentamethylmelamine (PMM), pentostatin, peptiochemio, plicamycin, prednimustine, probromane, procarbazine, profirmycin, paraplakin, prednisolone, prednisone, razoxane-rIFN $\alpha$ -2a, rubidazole, rIFN $\alpha$ -2b, rIFN $\beta$ -1b, rIFN $\gamma$ -1b, rIL-2, rTNF, semustine, SPG 827 (podophylline derivative), spirogermanium, streptonigrin, somatostatin, streptozocin, tamoxifen, taxol, thio-TEPA, 6-thioguanine, teniposide, testolactone, testosterone, 3-TGDR, rTNF, thyroglobulin, thyrotropin, trilostane, uracil mustard, VP-16, vincristine (VCR), vinblastine (VBL), verapamil, vindesine, vinorelbine, vitamin A acid, vitamin A analogues, zidovudine.

B) Peptides and peptoids.

C) Nucleic acids and nucleic acid derivatives.

D) Peptide nucleic acids (PNAs).

E) Hybrids of RNAs, DNAs, PNAs and derivatives.

The caspase activity can be measured both through the substrate turnover rate of fluorogenic or chromogenic substrates, or through the binding of specific markers, e.g., antibodies, Fab fragments, single-chain antibodies, aptamers (structure-binding nucleic acids), and/or other proteins having binding sites for either

unchanged (educts) or converted (products) caspase substrates. In particular, aminocoumarin-DEVD is suitable for use as a fluorogenic substrate.

Marker molecules which can specifically bind either educts or products of caspase reactions can comprise a dye portion, a colloidal metal (e.g., silver, gold), a radioactive isotope, and/or rare-earth metal chelates.

In particular, the accumulated caspase activity is measured no sooner than 10 h (especially from 24 to 48 h) after said at least one substance has been added to the sample, since only by then, even slowly acting cytostatic agents will have induced apoptosis. As apoptosis is a rather early indicator of the effectiveness of cytostatic agents, the test described is clearly quicker than for example, the MTT test with 4 days of incubation.

It is important that the caspase activities of equal cell numbers be compared upon apoptosis. Therefore, it is recommendable to standardize the extent of apoptosis for the total number of cells examined. This may be done, for example, by measuring the light absorption, scattered light, conductivity measurement, or microscopic counting. Standardization is necessary to be able to compare the extent of cellular damage caused by different cytostatic agents.

The method according to the invention is particularly suitable for the stratification of tumor diseases. According to the invention, a classification of the tumor diseases is enabled from which it can be seen which existing standard protocol for chemotherapy is appropriate. The test according to the invention permits to establish relatively quickly whether a particular tumor cell line will respond to a particular chemotherapeutic agent. Accordingly, it becomes possible to develop new protocols for the chemotherapy of tumor diseases by using the method according to the invention for the accumulated measurement of caspase activity as an indicator of the necrosis of tumor cells. Finally, the method according to the invention also allows to optimize an individual chemotherapy against tumor diseases by treating tumor tissue from a patient with chemotherapeutic agents and determining its response to these chemotherapeutic agents through apoptosis of the tumor cells according to the invention.

For example, a highly potent cytostatic agent which immediately stops the growth of cells and induces all cells to undergo apoptosis can exhibit significantly less apoptosis, due to the low number of cells, than is exhibited by a less potent cytostatic agent in the presence of which the cells can still proliferate at first and undergo apoptosis only later and to a low extent. Only standardization considers apoptosis in relation to the total number of cells and evaluates the more potent cytostatic agent correctly.

The chemosensitivity testing of cells can be performed to particular advantage with the aid of a kit. This kit comprises a sample support with several sample compartments, each sample compartment containing at least one substance. In addition, a standardized solution of a reagent for measuring caspase activity is also contained in each kit. In particular, the caspase substrate is aminocoumarin-DEVD in a lysis buffer. A caspase activity standard may also be contained in the kit. The sample support may be, for example, a commercially available microtitration plate. The selection of substances to be tested depends on the application; they may be provided ready to use in the compartments of the sample support either in solution or dried. The substances may be present either as dry substances, in solution, or in the presence of matrix substances, such as salts, buffers, carbohydrates, carboxylic acids, pyrimidines, inorganic or organic nanoparticles with diameters of up to 1  $\mu\text{m}$ .

Figure 1 shows the accumulated and conventionally measured caspase activities of Jurkat cells which were induced with actinomycin D for 4 h.

Figure 2 shows the different time courses of apoptosis in Jurkat and U937 cells upon induction by actinomycin D.

Figure 3 shows the chemosensitivity of U937 cells towards different cytostatic agents.

Figure 4 shows the chemosensitivity of HL60 cells towards different cytostatic agents.

Figure 5 shows the chemosensitivity of PBMNC towards daunorubicin.

Figure 6 shows the chemosensitivity of PBMNC towards Ara-C.

### **Examples**

#### **Example 1**

The caspase activity of 250 000 Jurkat cells in 100 µl of medium was determined after different incubation times with 1 µg/ml actinomycin D both conventionally and according to the method of accumulated caspase activity according to the invention. Thus, 100 µl of Jurkat cells ( $2.5 \times 10^6$ /ml) was incubated in the presence of actinomycin D at 5% CO<sub>2</sub> and 37 °C in RPMI medium with 10% FCS and Pen/Strep. After the incubation, the cells were centrifuged by a conventional method and washed with PBS. The pellet was resuspended in 200 µl of lysis buffer, incubated on ice for 10 min and frozen at -20 °C. The accumulated caspase activity was determined by mixing 100 µl of the cells incubated with actinomycin D, directly in suspension, with 100 µl of 2x lysis buffer. The lysate was also frozen at -20 °C. All lysates were simultaneously thawed, and aminocoumarin-DEVD was added. At room temperature, the release of fluorescent aminocoumarin by caspase was monitored every 5 min over a period of 2 h. From the linear increase of the signal, the slope was calculated as a measure of caspase activity. The spontaneous caspase activities of the Jurkat cells in the absence of actinomycin D, not shown in the Figure, increase with time, but remain lower than 4 min<sup>-1</sup>.

#### **Example 2**

The caspase activities of Jurkat and U937 cells were determined after different incubation times with actinomycin D. Thus,  $0.5 \times 10^6$  cells were respectively incubated with 1 µg/ml actinomycin D in cell culture plates (48 wells) in 1 ml of RPMI 1640 medium with 10% FCS and Pen/Strep for between 30 minutes and 16 hours. Subsequently, the cells were transferred to Eppendorf cups and washed twice with medium. The pellet was then resuspended in lysis buffer. After 10 minutes on ice, 50 µM aminocoumarin-DEVD substrate was added, and the



caspase activity was monitored at 37 °C every 5 min over a period of 2 hours. The fluorescence was excited at 355 nm, and the emission measured at 460 nm. The increase with time of the fluorescent emission is plotted against the incubation time with actinomycin D.

### Example 3

#### Chemosensitivity of tumor cell lines

The chemosensitivities of U937 and HL60 cells towards the cytostatic agents indicated in Figures 3 and 4 were tested in the stated concentration ranges. It can be seen from the Figures that the two tumor cell lines exhibit differently strong responses to cytostatic agents, as would be expected in the case of primary cells from patients.

The effect of four cytostatic agents on the cell line U937 was tested. Each data point represents the average from three independent experiments. As their concentration increases, the effectiveness of actinomycin D, daunorubicin and Ara-C also increases, while prednisolone causes no or but a slight apoptosis induction.

The maximum inducible caspase activity is relatively low for Ara-C, while it is significantly higher for daunorubicin, and highest for actinomycin D.

For comparison, the chemosensitivity of promyeloc HL60 cells is shown in Figure 2. In this case too, actinomycin D and daunorubicin induce higher caspase activities than Ara-C and prednisolone do. Prednisolone exhibits a maximum in caspase activity at about 700 ng/ml. At higher concentrations, the apoptosis signal decreases again. At the highest concentration measured, Ara-C appears not yet to have reached the maximum caspase activity inducible by this cytostatic agent.

#### Example 4

##### Chemosensitivity of PBMNC

For establishing the test for peripheral blood or bone marrow, isolated cells from the peripheral blood (PBMNC) of healthy volunteers were tested first, taking care that acute infections, as frequently encountered in the fall/winter season, could be excluded. Figures 5 and 6 illustrate the results of the studies with two cytostatic agents.

Figure 5 shows the effect of daunorubicin on the mononuclear cells of eight subjects (NP); in each case, the average  $\pm$  standard deviation from three independent experiments is shown. Caspase activity can be induced by  $> 50$  ng/ml daunorubicin; however, a plateau, i.e., an unchanging maximum caspase activity, is not reached for the subjects NP1 and NP5 to NP8, and only roughly so for the subjects NP2 to NP4. As compared to tested leukemia cell lines, the PBMNCs respond later to daunorubicin.

Figure 6 shows the effect of Ara-C on the mononuclear cells of subjects NP5 to NP8; in each case, the average  $\pm$  standard deviation from three independent experiments is shown. Caspase activity can be induced by  $> 200$  ng/ml Ara-C; however, a plateau, i.e., an unchanging maximum caspase activity, is not reached for any of the tested PBMNCs.

As compared with the data from the cell lines HL60 and U937 (Example 3), it is evident that the maximum inducible caspase activity is lower for those two cell lines.

CLAIMS:

1. A method for determining the chemosensitivity of cells towards at least one substance by measuring the apoptosis induced by said at least one substance, wherein the apoptosis is determined from the accumulated caspase activity of a sample comprising cells and a medium by adding said at least one substance to the sample followed by incubation, and measuring the accumulated caspase activity in the sample upon disruption of the cells without previously separating off the cells.
2. The method according to claim 1, wherein said cells are animal, including human, cells, especially leukemia cells, cells of solid tumors, cells of pathological organs, and/or reference cells, such as cells from organs other than the pathological ones, or cells from healthy regions of pathological organs.
3. The method according to any of claims 1 to 2, characterized in that pharmaceutically active substances, chemotherapeutic agents, environmental pollutants, peptides, nucleic acids or derivatives thereof, PNAs and/or nucleic acid hybrids are employed as said substances.
4. The method according to any of claims 1 to 3, wherein the caspase activity is measured through the substrate turnover rate of fluorogenic or chromogenic substrates, or through the binding of specific markers, such as antibodies, F<sub>ab</sub> fragments, single-chain antibodies, aptamers (structure-binding nucleic acids), and/or other proteins having binding sites for either unchanged (educts) or converted (products) caspase substrates.
5. The method according to any of claims 1 to 4, characterized in that said marker comprises a dye portion, a colloidal precious metal, a radioactive isotope, and/or rare-earth metal chelates.

6. The method according to any of claims 1 to 5, wherein the accumulated caspase activity is measured no sooner than 10 h, especially from 24 to 48 h, after said at least one substance has been added to the sample.
7. The method according to any of claims 1 to 6, wherein the caspase activity measured is standardized for the total number of the cells.
8. The method according to any of claims 1 to 7, wherein the measurement of caspase activity is employed for the stratification of tumor diseases, for developing new chemotherapies of tumor diseases, and/or for the optimization of an individual chemotherapy against tumor diseases.
9. A kit containing a sample support with sample compartments, each sample compartment containing at least one substance, and a standardized solution of a reagent for measuring caspase activity.
10. The kit according to claim 9, said substances being present as dry substances, in solution, or in the presence of matrix substances.
11. The kit according to any of claims 9 and/or 10, wherein said matrix substances are selected from salts, buffers, carbohydrates, carboxylic acids, pyrimidines, inorganic or organic nanoparticles with diameters of up to 1  $\mu\text{m}$ .

## Abstract

[illegible]

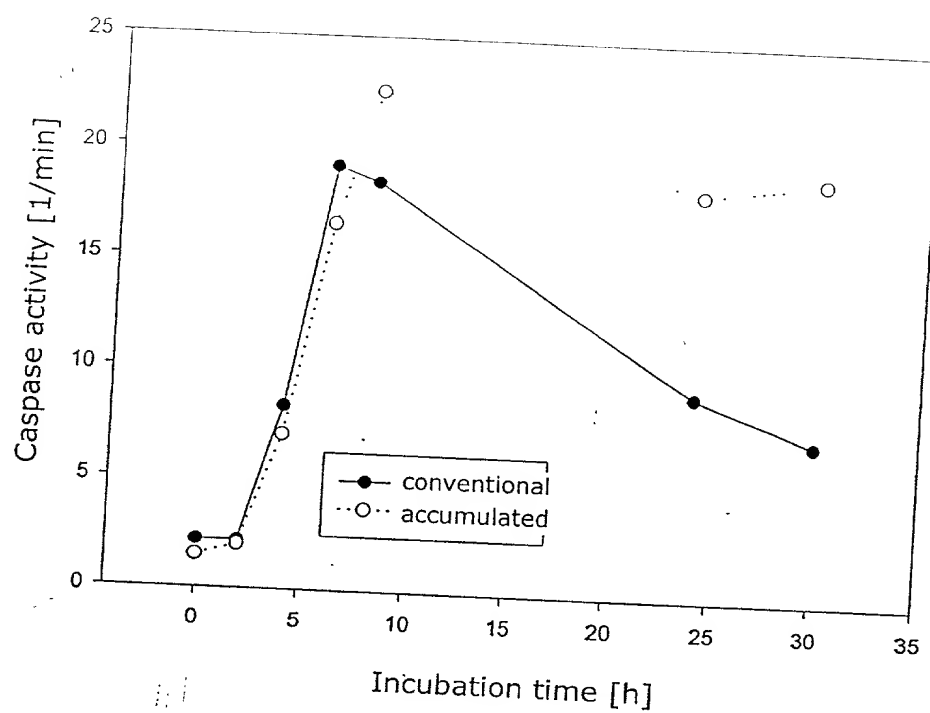


Figure 1

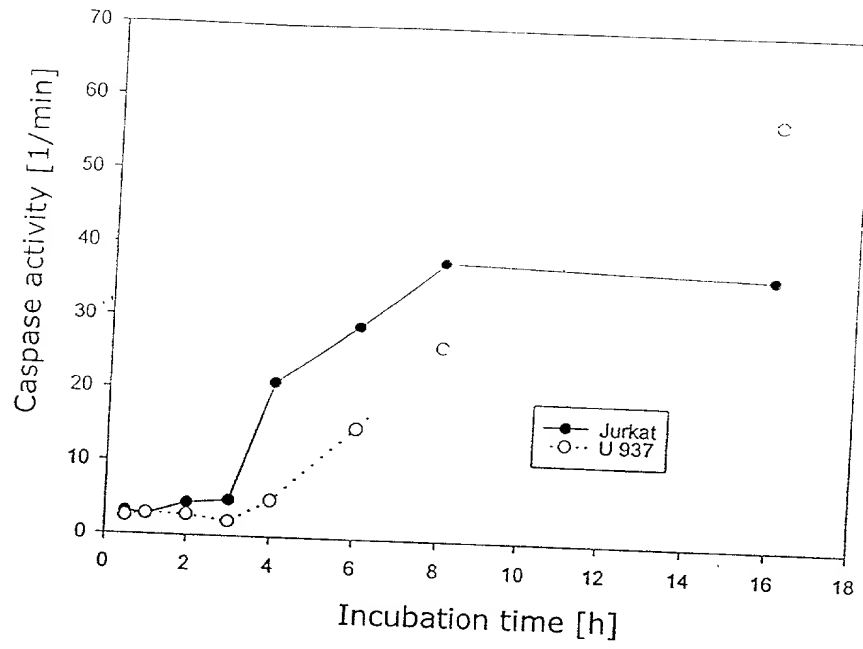


Figure 2

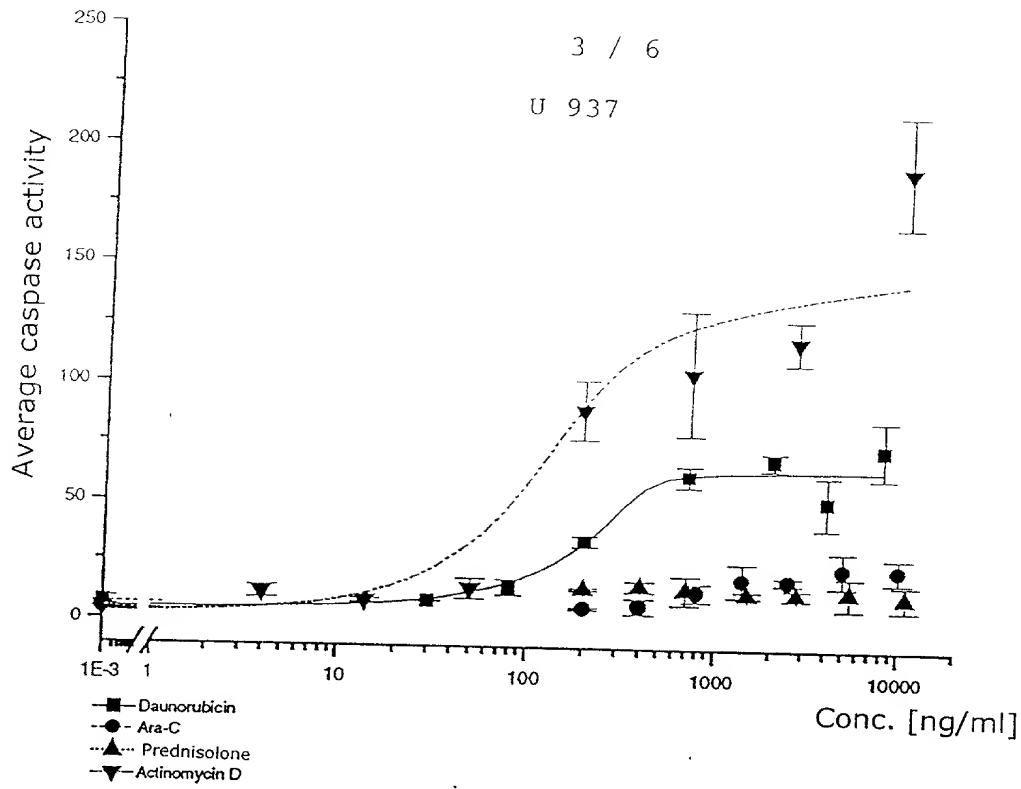


Fig. 3



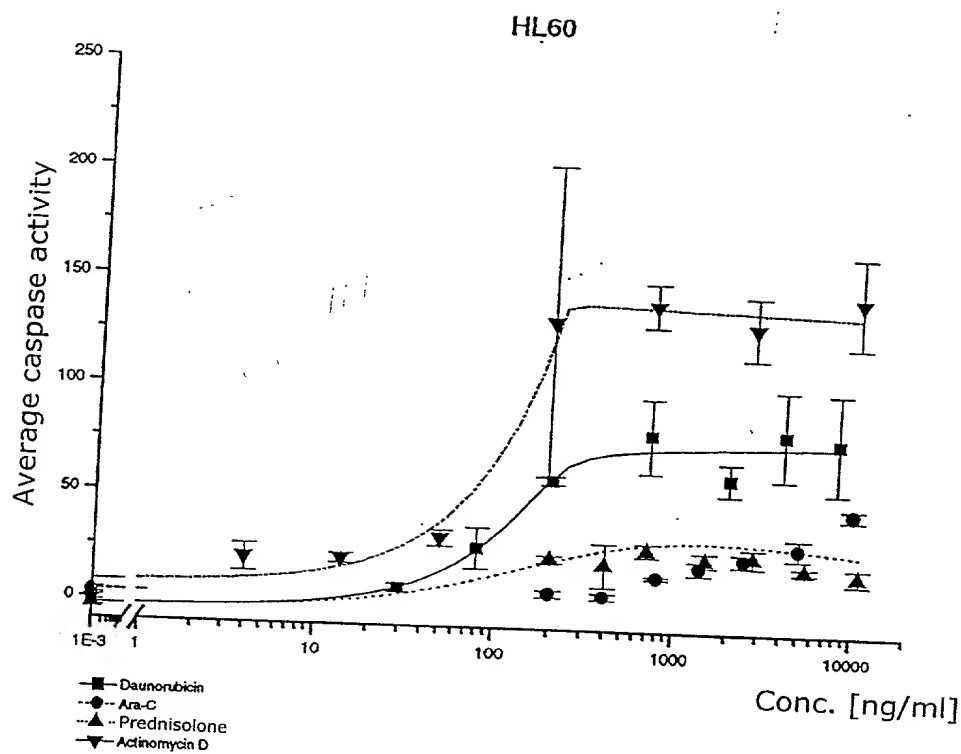


Fig. 4

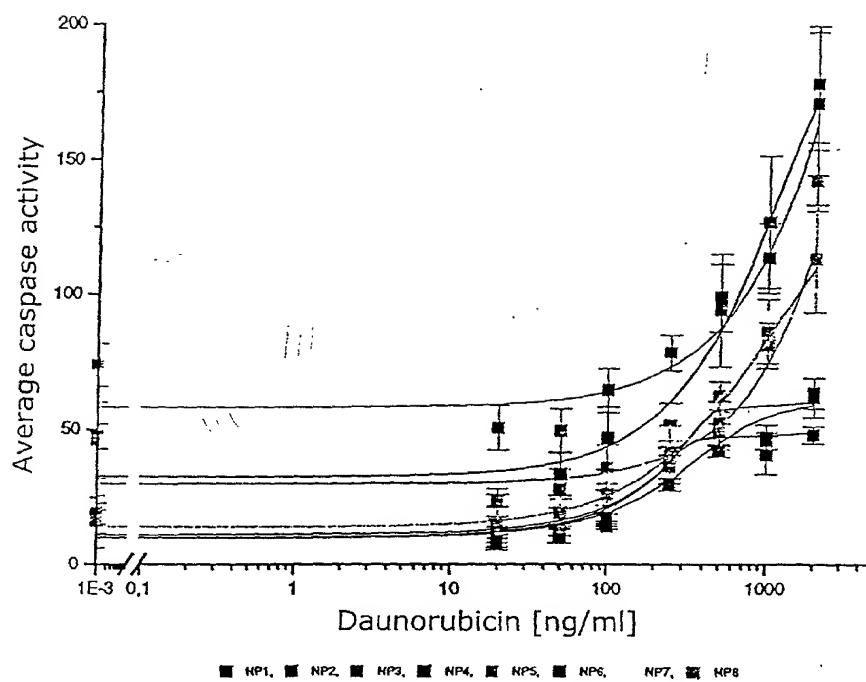


Fig. 5

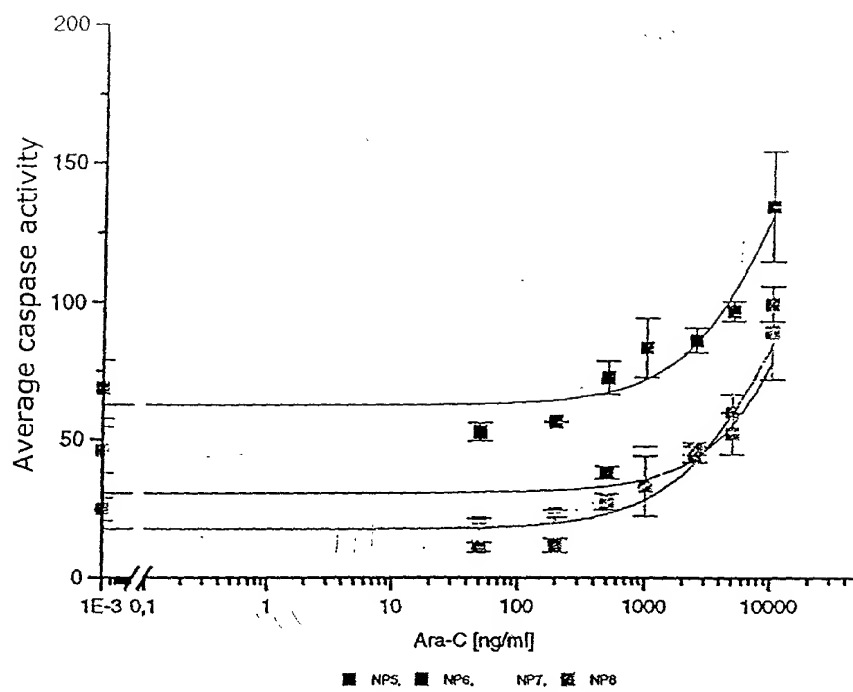


Fig. 6

DECLARATION  
AND POWER OF ATTORNEY  
U.S.A.

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ATTORNEYS' DOCKET NO.

ALL PATENTS, INCLUDING DESIGN  
FOR APPLICATION BASED ON PCT; PARIS CONVENTION;  
NON PRIORITY; OR PROVISIONAL APPLICATIONS

As a below named inventor, I declare that my residence, post office address and citizenship are stated below next to my name, the information given herein is true, that I believe that I am the original, first and sole inventor (if only one name is listed at 201 below), or an original, first and joint inventor (if plural inventors are named below at 201-203, or on additional sheets attached hereto) of the subject matter which is claimed and for which patent is sought on the invention entitled:

Chemosensitivity Measurement Using Caspase Activity

which is described and claimed in:

☒ PCT International Application No. PCT/EP 00/02174

filed March 13, 2000

☐ the attached specification

☐ the specification in application Serial No. \_\_\_\_\_

filed \_\_\_\_\_

(if applicable) and amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

For Foreign Application(s)

Priority Claimed

199 10 956.7

DE

March 12, 1999

☒ Yes

☐ No

(Number)

(Country)

(Day/Month/Year Filed)

99 108 495.5

EP

April 30, 1999

☒ Yes

☐ No

(Number)

(Country)

(Day/Month/Year Filed)

(Number)

(Country)

(Day/Month/Year Filed)

☐ Yes

☐ No

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

Application No. \_\_\_\_\_

Filing Date \_\_\_\_\_

Application No. \_\_\_\_\_

Filing Date \_\_\_\_\_

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.)

(Filing Date)

(Status: patented, pending, abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys (Registration No. ) to prosecute this application, receive and act on instructions from my agent, and transact all business in the Patent and Trademark Office connected therewith. HARVEY B. JACOBSON, JR. (20,851); D. DOUGLAS PRICE (24,514); JOHN CLARKE HOLMAN (22,769); MARVIN R. STERN (20,640); ALLEN S. MELSER (27,215); MICHAEL R. SLOBASKY (26,421); JONATHAN L. SCHERER (29,851); IRWIN M. AISENBERG (19,007); WILLIAM E. PLAYER (31,409); YOON S. HAM (45,307) and NATHANIEL A. HUMPHRIES (22,772)

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	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE OR COUNTRY ZIP CODE
203	FULL NAME * OF INVENTOR	FAMILY NAME	GIVEN NAME	MIDDLE NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
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I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201*	SIGNATURE OF INVENTOR 202*	SIGNATURE OF INVENTOR 203*
DATE <u>03/05/01</u>	DATE	DATE

☐ Additional inventors are named on separately numbered sheets attached hereto.

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